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Chemosensory responses in newborns of the fossorial earthsnake *Conopsis biserialis* (Serpentes: Colubridae) to potential invertebrate prey scents

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Use of chemosensory systems is common in terrestrial vertebrates as it helps to differentiate between numerous chemical signals present in their environments. Chemical cues processed by a sensory system (vomeronasal organ) are of overriding importance in many squamate reptile groups (Burghardt 1970, Halpern 1987). The use of chemical cues for recognition and prey discrimination has been extensively documented in snakes (e.g., Weldon et al. 1994, Theodoratus & Chiszar 2000, Weaver & Kardong 2010). Detection of prey-derived chemical cues by snakes is commonly associated with observed responses such as tongue flicking and latency period (Weaver et al. 2012). These behavioural expressions of chemosensation provide an opportunity to learn about heterospecific recognition and interest in different chemical cues emanating from potential prey (Cooper & Burghardt 1990). Ontogenetic changes in diet are frequently correlated with prey preferences in many generalist and large snakes (Mushinsky & Lotz 1980, Saviola et al. 2012). For their part, specialist snakes exhibit signs of innate chemical discrimination of specific prey types (Cooper & Secor 2007), with this discrimination being subject in some cases to genetic constraints and driven by the natural diet of each species (Alrnold 1980, Aubret et al. 2006). However, the innate components of this behaviour in newborn snakes could be replaced or modified by subsequent learning (Fuchs & Burghardt 1971, Arnold 1978) when prey is imprinted at the time of ingesting it for the first time in both natural conditions (Burghardt & Hess 1966) and in captivity (Loop 1970). Thus, information on predatory behaviour in neonates of many snake species contributes to understanding several aspects of their dietary ecology during their early ontogenetic stages. Whereas diet and predatory behaviour are known from several species of large snakes (mainly semi-aquatic and terrestrial snakes), little of this type of information exists for many other groups (e.g., fossorial and arboreal snakes).

The highly secretive and small (mean SVL: 200.3 mm) fossorial snake *Conopsis biserialis* is an ambush-hunting species with a geographic distribution in central and southern Mexico (Goyenechea & Flores-Villela 2006). It is commonly known as the two-lined Mexican earthsnake and information about its ecology and natural history is very limited (but see Castañeda-González et al. 2011, Raya-García et al. 2016). Frequently, these earthsnakes can be found in pine-oak forests surrounded by a mosaic of agricultural land and small, reforested patches (Vázquez & Quintero 2005, Castañeda-González et al. 2011). They spend most of their time sheltering under rocks or fallen trunks (Goyenechea & Flores-Villela 2006). The cryptic appearance of this species and its fossorial habits make observing it feeding in the field difficult , especially when it comes to its very small neonates. According to previous studies, *C. biserialis* is likely a dietary specialist predating upon fossorial insects (Ramírez-Bautista & Arizmendi 2004, Castañeda-González et al. 2011). However, its dietary preferences of distinct groups of invertebrate prey have never been assessed in either adults or neonates. Appropriate chemical discrimination of suitable/unsuitable invertebrate prey is even more relevant for the survival of small newborn earthsnakes, because a poor choice may result in exposure to toxins or at least poor energy intake, both of which may compromise survival and growth.

Here, we examined experimentally the recognition and discrimination of different invertebrate prey types by neonates of *C. biserialis* by chemical cues alone. In particular, we tested the hypothesis that a dietary specialist like the fossorial earthsnake *C. biserialis* at neonate size responds

readily to chemical cues from its preferred burrowing prey. We report on the typical chemosensory responses of predatory-naïve neonates of *C. biserialis* to chemical signals from some potential invertebrate prey.

Similar to previous studies of innate predatory behaviour in snakes with specialist diet (e.g., WELDON & SCHELL 1984, COOPER & SECOR 2007), we evaluated the behaviour of eight inexperienced specimens of *C. biserialis* born in captivity (2 May 2014) with mean snout–vent lengths (SVL) of 83.6 mm (range = 79–85 mm) and mean body masses of 1.2 g (range = 1.2–1.5 g) from one female. Our sample size is limited because the fossorial habits of *C. biserialis* hindered the capture of additional gravid females. Therefore, the rarely observed birth of a litter in captivity presented an infrequent opportunity to research innate behavioural expressions (COOPER & SECOR 2007). The mother had been collected on 23 March 2014, at Ichaqueo, municipality of Morelia, Michoacán, México. This female had a SVL of 280 mm and a body mass of 24 g. In snakes with generalist diet, increased tongue-flicking is a common response to chemical cues from potential prey (GREENBAUM 2004, COOPER 2008). Therefore, the probability that a single litter of a fossorial snake with a specialist diet would exhibit strong responses only to chemical cues from one type of prey simply by causality is extremely low (COOPER & SECOR 2007).

The female and her neonates were kept under terrarium conditions in the herpetology laboratory of the Instituto de Investigaciones Sobre los Recursos Naturales in the city of Morelia, Michoacán, Mexico (2000 m a.s.l.). Ethical responsibilities were strictly observed. The female and her neonates were returned to their site of capture after our trials had been completed.

The newborns were placed individually in translucent plastic containers (35 × 25 × 15 cm) with a tight lid and small holes for ventilation. Each container had coconut fibre substrate and a bowl offering water *ad libitum*. During and before each experiment, the snakes had never been fed or exposed to any prey. The containers of each snake were kept in a natural photoperiod of 12:12 hours (day:night) at a temperature of 23–24°C in a laboratory room isolated from noise and minimum human traffic. The snakes were monitored under these conditions for two days and their behavioural responses were noted even before their first moult.

The housing containers for the snakes were also used as experimental boxes when prey chemical stimuli were presented on 15-cm cotton swab applicators (COOPER & BURGHARDT 1990, COOPER 1998). We used the scents of four potential invertebrate prey items, earthworms (*Lumbricus terrestris*), adult beetles (*Tenebrio molitor*), crickets (*Acheta domesticus*), and ants (*Atta* sp.). Distilled water (odourless substance) was used as a control item and to gauge a baseline of predatory behaviour (COOPER & BURGHARDT 1990). Pungency controls were not used to avoid possible invasive scent effects on chemoreceptors of the newborn snakes. All four invertebrate prey types (ant, beetle, earthworm and orthopteran) have been reported as forming part of the diet of adult *C. biserialis* (URIBE-

PEÑA et al. 1999, RAMÍREZ-BAUTISTA & ARIZMENDI 2004, CASTAÑEDA-GONZÁLEZ et al. 2011). The scents or prey chemical stimuli were obtained by following the basic methodology of BURGHARDT (1966, 1968). This technique involves placing one or more prey individuals in distilled water previously heated to 50°C for one minute, at a proportion of 10 ml water per 1.5 g of prey. Subsequently, the prey animal or animals is/are removed from the container and the remaining liquid is centrifuged at 2500 rpm for ten minutes. The supernatant liquid is then poured into sterilized vials and kept refrigerated until it is to be used. All chemical stimuli swabs and setting up the experiments were effected simultaneously on the same day.

The procedure involved dipping the cotton swab in the prey chemical stimuli liquid and carefully approximating the swab at a distance of 1 cm in front of the snake nose for 60 seconds (COOPER & BURGHARDT 1990). During this time, we recorded two variables, i.e., the total number of tongue-flicks and the latency period (time in seconds before first tongue-flick) displayed by the snakes in response to each chemical stimulus (WEAVER et al. 2012). Their responses were filmed with a video camera (Panasonic SDR-H101) on a tripod, and focused on the inside the experimental box. In order to minimize possible effects of disturbance on snake's behaviour, trials commenced five minutes after the lid had been removed from their containers (COOPER & SECOR 2007, WEAVER & KARDONG 2010). Individuals were selected randomly and evaluated during the afternoon (14:00–16:00 h) in a closed room with dim lighting and a temperature of 27–28 °C. Each chemical stimulus (vomodor) was presented only once to the same individual in random order. Only one trial was conducted per day, resulting in five trials with each of the eight snakes on five days. Each experimental container was afterwards cleansed with commercial detergent and rinsed with distilled water to remove possibly lingering odours from the previous experiment.

Due to the lack of independency, normality, and variance homogeneity in the data, we used nonparametric Friedman two-way analyses of variance (ZAR 1996) to evaluate the effect of prey scents on latency period and rate of tongue flicks in all neonate earthsnakes. Additionally, a post hoc analysis of multiple paired comparisons was conducted using Tukey's honestly significant difference (HSD) tests. All statistical analyses were performed with the interface Rstudio in R software version 3.3.2 (R Development Core Team 2013).

All snakes responded with tongue flicks and latency periods to the five trials (four chemical stimuli and the control dummy), with the most common response displayed being tongue flicks (36 of 40 trials, 90%). None of the neonates attacked the cotton swab during the experiments. There was a significant effect of the chemical stimuli on tongue flicking ($\chi^2 = 12.20$, df = 4, P < 0.01). The number of tongue flicks to beetle scent was significantly different from that to the water-soaked control dummy (Tukey's test P < 0.001), but also different from ant, cricket, and earthworm scents (P < 0.01, to all; Fig. 1). There were no significant differences in the number of tongue flicks between ant, cricket and earthworm scents (P = 0.99, to all; Fig. 1).

Finally, there was a significant effect of chemical stimuli on latency periods ($\chi^2 = 15.77$, df = 4, P < 0.01), which was longer to distilled water (control) than to all other scents evaluated, and there were significant differences between the beetle scent and distilled water (P < 0.001; Fig. 2) and between cricket and beetle scents (P < 0.05; Fig. 2).

Our results suggest that neonates of the two-lined Mexican earthsnake *C. biserialis* possess the chemosensory ability to recognize invertebrate prey. Based on the obtained results, predatory-naïve neonates display a longer lasting response to chemical cues of a beetle than to those of other invertebrate prey. Beetles of the order Coleoptera have been reported as forming part of the diet of *C. biserialis* (Uribe-Peña et al. 1999, Ramírez-Bautista & Arizmendi 2004). Apterism and high diversity of geophyle beetles renders them of interest to a a large number of predators (Carrara 2011). Beetles of small size, with fossorial habits, and their increased activity during periods of reduced insolation
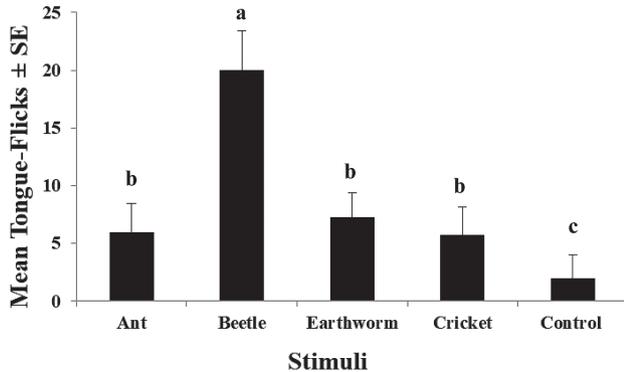

Figure 1. Mean number of tongue flicks (± SE) displayed by neonates of *Conopsis biserialis* in response to chemical scents from potential invertebrate prey and a water control dummy. The letters above the bars indicate significant differences between invertebrate scents with identical letters indicating no significant differences.
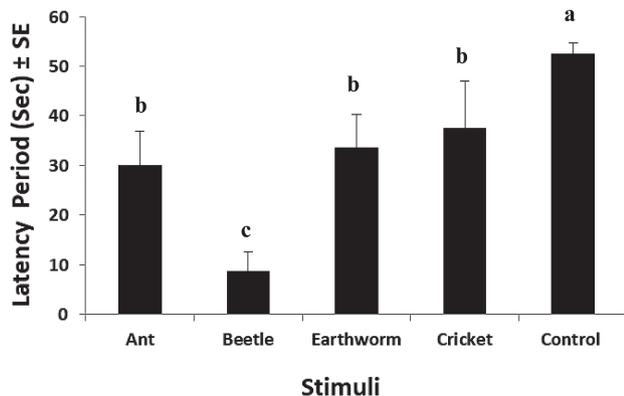

Figure 2. Means of latency periods (± SE) displayed by neonates of *Conopsis biserialis* in response to chemical scents from potential invertebrate prey and a water control dummy. The letters above the bars indicate significant differences between invertebrate scents with identical same letters indicating no significant differences.

(Cloudsley-Thompson 2001) probably make them easy prey to furtive reptiles. The ability to sense and discriminate beetle chemical cues from others is probably indicative of small and fossorial beetles being an important prey item in the early diet of *C. biserialis*. The latency period and tongue flicks to ant, cricket and earthworm chemical cues did not vary significantly, and according to Weaver & Kardong (2010) this is a result of snakes responding to stimuli as novel scents, but are not detected like potential prey chemicals due to their low sensory chemical specificity.

Although several small, fossorial and myrmecophagous blindsnakes are known to detect scent trails of worker ants (Watkins et al. 1967, Webb & Shine 1992), it appears that ant scents are unimportant prey chemical cues to neonates of the fossorial earthsnake *C. biserialis*, which is consistent with the adults' diet in which ants do not play a role as prey (Castañeda-González et al. 2011). Visual cues were not evaluated in this study, because we considered them of lesser importance due to the fossorial lifestyle of earthsnakes (Goyenechea & Flores-Villela 2006).

The individuals used in this experiment had never been exposed to any type of prey before our trials. Therefore, their chemosensory responses were not a reflection of previous experiences with prey under both captive and natural conditions (Burghardt et al. 2000). The earthsnake *C. biserialis* is considered an oligophagous specialist, predating upon a few burrowing insects (Castañeda-González et al. 2011), therefore their chemosensory responses might constitute a specific component of innate prey recognition (Burghardt 1968, Cooper & Secor 2007). Interestingly, the neonate snakes in our experiments did not attack the scented cotton swabs, contrary to adult and juvenile individuals that have previously been observed biting swabs with invertebrate chemical stimuli (Raya-García 2016). The size, defence capabilities, or toxicity of prey and other opportunities to gain experiences probably play a crucial role in developing predatory behaviours of many snakes during their early ontogenetic stages by reinforcing or adapting these instinctive responses through learning (Arnold 1978, Terrick et al. 1995, Waters & Burghardt 2005). Field studies are necessary to generate more information about the predatory and foraging habits of fossorial and secretive snakes.

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